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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicant: Madison et al.

Title: TARGETED THERAPEUTIC OR  
DIAGNOSTIC AGENTS AND  
METHODS OF MAKING AND  
USING SAME

Appl. No.: 09/091,578

Filing Date: June 19, 1998

Examiner: Schwadron, Ronald B.

Art Unit: 1644

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**Declaration of Drs. Edwin L. Madison, Ph.D. and Jeffrey W. Smith, Ph.D.**

1. I, Edwin L. Madison, received a Ph.D. degree in biochemistry from The University of Texas - Southwestern Medical Center (UTSWC) in 1990. Upon graduation, I accepted a faculty position in the Department of Biochemistry at UTSWC. In 1993, I moved to The Scripps Research Institute (SRI) as an Assistant Professor of Vascular Biology. In 1995, I was promoted to Associate Professor of Vascular Biology at SRI, and in 1998 I accepted positions as Director of Molecular Biology at Corvas International, Professor of Vascular Biology at The Torrey Pines Institute for Molecular Studies (TPIMS), and Adjunct Associate Professor of Cell Biology at SRI. In 1999, I was promoted to Senior Director of Molecular Biology, and in 2000, I became Executive Director of Molecular Biology. In 2001, I was promoted to my current position, Vice President of Biological Research, at Corvas International, and I continue to maintain my positions as Professor of Vascular Biology at TPIMS and Adjunct Associate Professor of Cell Biology at SRI. I have achieved an international reputation in the fields of protein structure-function and development of new technologies in the area of molecular

biology. I have presented four invited Plenary and State-of-the-Art lectures at international scientific meetings, and I have served as chairperson for 14 scientific sessions at international meetings. I have also served on the Editorial Board of two scientific journals. My current CV lists 43 publications in the primary scientific literature, and all of these manuscripts address protein structure/function relationships, ligand binding, or molecular mechanisms or describe new molecular biological or biochemical techniques. Eight of these manuscripts describe uses of phage display technology, and ten of these scientific papers report results of successful protein engineering experiments.

2. I, Jeffrey W. Smith, received a B.A. degree in biology from the University of California at Santa Barbara (1983) and a Ph.D. degree from in biology from the University of California at Irvine (1987). I performed post-doctoral research at the Scripps Research Institute, where I was appointed to the faculty in 1992. In 1995 I accepted the position of Program Leader (Department Chair) at the Burnham Institute in La Jolla, California. I currently continue to work at the Burnham Institute where I serve as Associate Scientific Director. I am an author or co-author on 55 peer-reviewed published papers, more than 30 of which report findings in the area of protein-structure-function relationships, and 8 of which deal specifically with protein design and engineering by phage display.

3. We are co-inventors of U.S. patent application Serial No. 09/091,578, filed October 6, 1998, titled "Targeted Therapeutic or Diagnostic Agents and Methods of Making and Using Same" (hereinafter "the Application").

4. The invention described in the application involves a therapeutic agent having an entity with a therapeutic property and an exogenous surface loop that specifically binds to a target. The therapeutic entity retains its therapeutic property and the surface loop retains a

specific binding characteristic for a target. In one embodiment the therapeutic entity is tissue plasminogen activator (t-PA) and the exogenous surface loop is a heavy chain CDR3 of monoclonal antibody Fab-9. Loop-grafted tissue plasminogen activator (LG-t-PA) is a targeted therapeutic that directs tissue plasminogen activator to integrins present in an evolving arterial thrombus (i.e., blood clot) using the grafted loop as the targeting mechanism. When we planned the experiment to create LG-t-PA, we did not know whether an exogenous surface loop, removed from its normal structural context (in this case, the complementarity determining region (CDR) of an antibody) would retain its function when placed in the structurally disparate context of a domain of t-PA. In fact, we suspected that such a grafting might not result in a successful targeting molecule for the reasons described below.

5. A key aspect of this invention is the grafting of a protein surface loop into another protein. The claims recite that the grafted loop retains a specific binding characteristic for a target, and that the therapeutic property of the recipient protein is preserved. At the time that this invention was conceived and reduced to practice, there was no indication from published art as to whether it would be possible to create such a molecule and retain the binding characteristics of a grafted loop with the therapeutic property of the recipient protein.

6. Specifically, the invention involves protein surface loops, which are flexible loop structures of 4-20 amino acids that lie between two stretches of secondary structure, or that connect a domain of secondary structure and a terminus of the native protein. At the time of the invention, no work had been done on loop optimization and grafting in proteins other than antibodies. Thus, information related to loop grafting came primarily from the antibody field.

7. The antigen binding site of an antibody is comprised of six surface loops called complementarity determining regions (CDRs). In the early 1990s, there was a great deal of

interest in exchanging multiple CDRs between antibodies so that mouse monoclonal antibodies could be "humanized." One hypothesis at the time suggested that this could be accomplished by simply exchanging multiple CDRs from one antibody to another. But this strategy often it failed to produce antibodies that retained the binding characteristics of the grafted loops (Studnicka GM, Soares S, Better M, Williams RE, Nadell R, Horwitz AH "Human-engineered monoclonal antibodies retain full specific binding activity by preserving non-CDR complementarity-modulating residues." *Protein Eng* (1994) Jun;7(6):805-14). Thus, the parameters involved in grafting a loop from one antibody to another were not understood.

8. The study of Smith and Barbas tested whether a loop from an antibody CDR (i.e., HCDR3 of Fab-9) that was known to mediate binding to a selected target could be removed from the antibody context and retain its binding affinity as a linear peptide. This study also examined whether cyclizing such a peptide to force it into the conformation of a loop might allow it to retain the binding characteristics it exhibited in the parent antibody (i.e., Fab-9). The authors of this study found that neither the linear nor the cyclic CDR-derived peptide retained the binding characteristics that the CDR exhibited in Fab-9 (Smith et al, "Building synthetic antibodies as adhesive ligands for integrins." *J. Biol. Chem*, (1994), 269: 32788-32795). The study showed that both the linear and cyclic versions of the CDR-derived synthetic peptides possessed the same low binding affinity for antigen (in this case integrins). Therefore, cyclization of the peptides into an exogenous "loop" failed to either improve target binding or to recapitulate the character of the exogeneous sequence in the context of a CDR region within the parent antibody. Thus, these findings indicated that the optimized loop did not retain its full binding function in the absence of the antibody scaffold. Consequently, this information also failed to provide an indication that a surface loop could be successfully grafted from one protein to another.

9. Thus, the information available in the public domain did not provide guidance as to whether a loop could be grafted from one protein scaffold to another. Indeed, the study of Smith and Madison was the first to test this concept (Smith J.W., Tachias, K. and Madison, E.L., "Protein Loop Grafting to Construct a Variant of Tissue-type Plasminogen Activator That Binds Platelet Integrin." *J. Biol. Chem.*, (1995), 270: 30486 - 30490). In order to provide a highly rigorous test of this broad concept, a recipient protein (t-PA) was selected that was completely unrelated and structurally disparate from the donor protein (the antibody Fab-9).

10. Surprisingly, we found that the loop-grafted t-PA retained its full therapeutic property, and the inserted surface loop from Fab-9 was unaltered in its specific binding characteristic for integrin. Because of the highly stringent nature of this experiment, we realized that loops do indeed retain their binding function when grafted from one protein domain to another. As stated on pp 30489 of Smith et al. (1995), "The affinity of the modified tPA for this integrin was also high, with an apparent  $K_D$  of 1.8 nM, similar to the  $K_D$  of 1.7 nM exhibited by Fab-9." Therefore, this study showed that grafting of an optimized loop between structurally distinct protein scaffolds was possible, while retaining the specific binding character of the grafted loop and the therapeutic property of the recipient protein. The t-PA utilized in this experiment has a protein scaffold that is very different from the scaffold environment where the surface loop was taken from (Fab-9). The fact that the loop graft succeeded in such a different scaffold environment contributed to our realization that surface loops could be grafted into proteins generally. Thus, we realized that surface loops with specific binding characteristics could be grafted into a wide variety of proteins, since it had been successfully accomplished with a loop from a very different scaffold environment.



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11. Since the publication of our report, other studies have proven our realization to be correct. These other studies report success in grafting loops into different recipient proteins. For example, Dennis et al., "Albumin binding as a general strategy for improving the pharmacokinetics of proteins." *J. Biol. Chem.* 277(38):35035-43 (2002), describe the creation of a library of cysteine-constrained loops displayed on the surface of bacteriophage. From this library of loops the authors selected a number of albumin-binding loops. Some of these loops are inserted into single chain antibodies (scFv). The resulting scFvs retain their affinity for the original antigen, and acquire the albumin-binding properties of the original loop. Thus, surface loops can be grafted into a variety of protein scaffold environments and retain their specific binding characteristics, even as the therapeutic entity they are grafted into also retains its therapeutic property.

12. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Capital Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

5/30/03  
Date

Edwin L. Madison  
Edwin L. Madison, Ph.D.

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Date

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Jeffrey W. Smith, Ph.D.

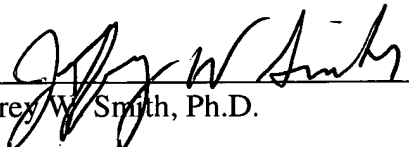
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Edwin L. Madison, Ph.D.

  
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